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(54) **Process for transforming a human insulin precursor to human insulin.**

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BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 38, no. 2, 1970,pages 284-289; B.H. FRANK et al.: "Interaction of zinc with proinsulin"

JOURNAL OF CLINICAL INVESTIGATION, vol. 76, October 1985, pages 1398-1405, TheAmerican Society for Clinical Investigation, Inc.; B.D. GIVEN et al.:"Biochemical and clinical implications of proinsulin conversion intermediates"

BIOCHEMISTRY, vol. 11, no. 26, 1972, pages 4926-4931; B.H. FRANK et al.:"Physical studies on proinsulin. A comparison of the titration behaviour of thetyrosine residues in insulin and proinsulin"

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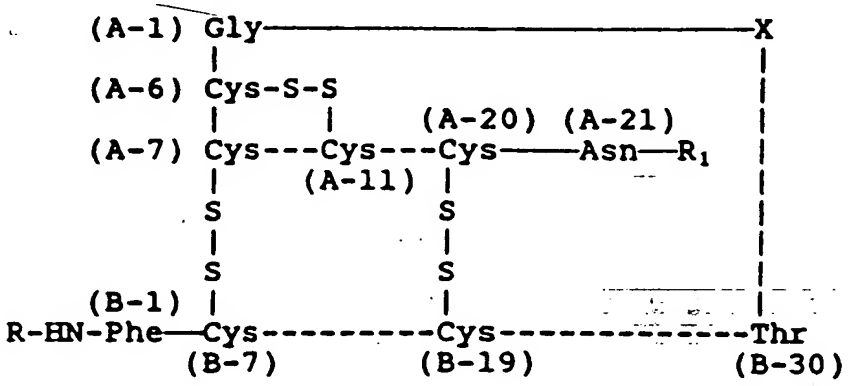
Description

The ability to convert proinsulin to insulin using trypsin and carboxypeptidase B has been recognized for several years [see, e.g., Kemmler, W., Clark, J.L., Borg, J. and Steiner, D.F., Fed. Proc. 30 (1971) 1210; Kemmler, W., Peterson, J.D., and Steiner, D.F., J. Biol. Chem., 246 (1971) 6786-6791]. An ongoing difficulty with this conversion methodology has been and continues to be the presence of substantially large amounts of difficultly-removable by-products in the reaction mixture. Specifically, in the conversion of human proinsulin to human insulin, a large amount (about 4-6%) of Des-Thr(B30) human insulin [Des-Thr(B30)-hI] is formed. This by-product, differing from human insulin by the absence of a single terminal amino acid, is, if capable of being separated from the product mixture at all, separated only by difficult and cumbersome methodology.

With the advent of recombinant DNA technology, for the first time large amounts of human proinsulin have become a reality. In using the human proinsulin as an intermediate in the production of insulin, a solution to the Des-Thr(B30)-hI impurity problem has become mandatory. Either one could seek ways to achieve purification of the human insulin from the contaminating Des-Thr(B30)-hI or seek a conversion process, the conditions of which minimize formation of the latter. For example, the implication of zinc in the conversion of proinsulin to insulin is mentioned in BBRC 38 (1970) 284-9.

It is to a new process for converting a human insulin precursor to human insulin with minimal formation of Des-Thr(B30)-hI that the present invention is directed.

Thus, this invention is directed to a process for converting a human insulin precursor to human insulin, such precursor having the formula



in which R is hydrogen, a chemically or enzymatically cleavable amino acid residue, or a chemically or enzymatically cleavable peptide moiety having at least two amino acid residues;

R_1 is OH, Arg-Y, or Lys-Y in which Y is OH, an amino acid residue, or a peptide moiety having at least two amino acid residues;

the moiety from A-1 to A-21 is the human insulin A-chain; the moiety from B-1 to B-30 is the human insulin B-chain; and X is a moiety which is joined to the insulin A-chain at the amino group of A-1 and to the insulin B-chain at the carboxyl group of B-30, which moiety can be enzymatically cleaved from and without disruption of both the A-chain and the B-chain, which comprises treating such human insulin precursor with trypsin and carboxypeptidase B in an aqueous medium containing per mole of human insulin precursor from about 0.1 to about 10 moles of one or more metal ions of those metals having Atomic Numbers 21 to 34, 39 to 52, 57 to 84, and 89 to 92.

As indicated, the process of this invention represents an enhancement of the recognized conversion of proinsulin to insulin using trypsin and carboxypeptidase B. The process is applied to human insulin precursors of the foregoing formula, the most preferred of which is human proinsulin itself.

As used herein, the term "human insulin precursor" refers to a molecule which (1) contains the human insulin A-chain and the human insulin B-chain, (2) has at least three disulfide bonds represented by a joining of the sulfurs of each of the Cys moieties located in the A- and B-chains at (a) A-6 and A-11, (b) A-7 and B-7, and (c) A-20 and B-19, respectively, and (3) has a removable connecting moiety which is joined to the insulin A-chain at the amino group of A-1 and to the insulin B-chain at the carboxyl group of B-30.

The group R is hydrogen, an amino acid residue, or a peptide moiety having at least two amino acid residues. In those instances in which R is an amino acid residue or a peptide moiety, R is a group which is cleavable from the insulin precursor product without loss of the integrity of the residual insulin structure.

Any of a wide variety of amino acid residues or peptide moieties qualify within the definition of the group R. Examples of cleavable amino acid residues are basic amino acids such as arginine (Arg) or lysine (Lys) as well as peptide moieties terminating at the carboxyl by such amino acid residues. These are recognized as susceptible to cleavage upon treatment with the proteolytic enzyme trypsin. Another example of a cleavable amino acid residue is methionine (Met) as well, again, as a peptide moiety having Met at its carboxy terminal. These can be removed by treatment with cyanogen bromide. A further example is tryptophan (Trp) or a peptide moiety containing Trp at its carboxy terminal. This is removed upon treatment with N-bromosuccinimide.

The group R_1 is hydroxyl, arginine, lysine, or a peptide having arginine or lysine at its amino terminus. When R_1 is arginine, lysine, or a peptide having either of these residues at its amino terminus, the amino acid or peptide will be cleaved under the conditions of the process of this invention with formation of a product in which R_1 is hydroxyl.

The connecting moiety, X, of the insulin precursor can be any of a wide range of structures. Preferably, the moiety X is a polypeptide. The polypeptide generally has at least 2 and preferably from about 2 to about 35 and most preferably from about 6 to about 35 amino acid residues. The moiety X is joined to the A-chain at the amino group of A-1 and to the B-chain at the carboxyl group of B-30. Most preferably, the connecting moiety, X, when it is a peptide, is the natural connecting peptide of human proinsulin, such connecting peptide having the formula:

-Arg-Arg-Glu-Ala-Glu-Asp-Leu-Gln-Val-Gly-
Gln-Val-Glu-Leu-Gly-Gly-Gly-Pro-Gly-Ala-
Gly-Ser-Leu-Gln-Pro-Leu-Ala-Leu-Glu-Gly-
Ser-Leu-Gln-Lys-Arg-.

Although it is preferred to use the natural connecting sequence, as indicated above, much shorter peptide sequences can be used for the connecting peptide. The only requirements are (1) that they be of sufficient length to permit proper disulfide bond formation between the A- and B-chains, and (2) that they be cleavable from the insulin precursor with accompanying insulin formation. A typical dipeptide which can be used is -Arg-Arg-. In addition, modifications of the foregoing dipeptide having the formula -Arg-X^1-Arg- in which X^1 represents at least one amino acid residue can be readily employed. Highly preferred connecting peptides are -Arg-Arg-Lys-Arg- as well as longer chain peptides having the structure -Arg-Arg-X^2-Lys-Arg- in which X^2 is at least one amino acid residue and preferably at least two amino acid residues. These latter, of course, include the natural connecting peptide.

The process of this invention is conducted in an aqueous medium. The term "aqueous medium" requires the presence of water; it does not, however, preclude the presence of water-miscible organic solvents such as methanol, ethanol, acetone, N,N-dimethylformamide, and the like. The human insulin precursor is present in the medium at a concentration of up to about 20 mM. Preferably, the human insulin precursor concentration is substantially lower, ranging generally from about 0.1 mM to about 10 mM; more preferably, from about 0.5 to about 5 mM; and most preferably, from about 1 to about 3 mM.

The conversion is carried out at any of a wide range of temperatures, generally from about 0 °C to about 40 °C. Preferably, the reaction is conducted at a temperature of from about 4 °C to about 25 °C, and, most preferably, from about 10 °C to about 15 °C.

The pH of the reaction mixture can range anywhere from about 4 to about 12. However, best results are obtained by careful pH control such that the reaction is conducted at a pH in the range of from about 6 to about 9, preferably from about 7 to about 8, and, when precisely controlled, from about 7.2 to about 7.6.

pH Control generally is assisted by the use of a buffering agent. Any of a wide range of typical buffers can be employed. Examples of suitable buffers are TRIS [tris(hydroxymethyl)aminomethane], ethylene diamine, triethanolamine, glycine, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), and the like.

The amount of trypsin and carboxypeptidase B that generally is used is related both as between the two enzymes and to the amount of human insulin precursor. The enzymes can be incorporated in the reaction mixture either in solution or, using recognized techniques, can be immobilized on a suitable support and thereby made available in the reaction medium.

On a weight:weight basis, carboxypeptidase B generally will be present in an amount relative to the human insulin precursor of from about 1:10 to about 1:5,000; preferably, from about 1:500 to about 1:3,500;

and, most preferably, from about 1:1,000 to about 1:3,000.

On a weight:weight basis, trypsin generally will be present in an amount relative to the human insulin precursor of from about 1:20 to about 1:250,000; preferably, from about 1:300 to about 1:20,000; and, most preferably, from about 1:5,000 to about 1:15,000.

The ratio of carboxypeptidase B to trypsin in the reaction mixture also represents an important parameter. Generally, on a weight basis, the ratio, carboxypeptidase B to trypsin, will be from about 1:1 to about 10:1, and, preferably, from about 2:1 to about 5:1.

The key discovery which forms the basis of this invention resides in the finding that the presence of a defined amount of one or more of a wide range of metal ions substantially diminishes the amount of Des-Thr(B30)-hI formed during the reaction.

Although certain metal ions are highly preferred, it has been discovered that a wide range of such ions are useful. Metal ions that can be employed are those of the following metals: scandium (Sc), titanium (Ti), vanadium (V), chromium (Cr), manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), gallium (Ga), germanium (Ge), arsenic (As), selenium (Se), yttrium (Y), zirconium (Zr), niobium (Nb), molybdenum (Mo), technetium (Tc), ruthenium (Ru), rhodium (Rh), palladium (Pd), silver (Ag), cadmium (Cd), indium (In), tin (Sn), antimony (Sb), tellurium (Te), lanthanum (La), cerium (Ce), praseodymium (Pr), neodymium (Nd), promethium (Pm), samarium (Sm), europium (Eu), gadolinium (Gd), terbium (Tb), dysprosium (Dy), holmium (Ho), erbium (Er), thulium (Tm), ytterbium (Yb), lutecium (Lu), hafnium (Hf), tantalum (Ta), tungsten (W), rhenium (Re), osmium (Os), iridium (Ir), platinum (Pt), gold (Au), mercury (Hg), thallium (Tl), lead (Pb), bismuth (Bi), polonium (Po), actinium (Ac), thorium (Th), protactinium (Pa), and uranium (U).

Although ions of any of the foregoing metals can be used in the process of this invention, highly preferred subclasses of narrowing scope and thus increased preference are as follows:

(1) chromium, molybdenum, tungsten, mercury, antimony, bismuth, nickel, iron, cobalt, zinc, cadmium, copper, tin, lead, europium, uranium, platinum, and manganese.

(2) nickel, iron, cobalt, zinc, cadmium, copper, tin, lead, europium, uranium, platinum, and manganese.

(3) nickel, zinc, cobalt, and cadmium.

(4) nickel and zinc.

(5) nickel.

In accordance with the process of this invention, ions of one or more of the foregoing metals are added to the human insulin precursor reaction mixture. The amount of ion from the foregoing metals in the aggregate present in the reaction mixture ranges from about 0.1 to about 10 moles per mole of human insulin precursor. The actual amount used preferably is at the lower end of the foregoing range, generally being from about 0.1 to about 2 moles per mole of human insulin precursor. Most preferably, the amount is from about 0.3 to about 1 mole per mole of human insulin precursor, and, ideally, from about 0.33 to about 0.6 moles per mole of human insulin precursor.

The conversion reaction normally is conducted for a period of from about 2 hours to about 48 hours, usually from about 8 hours to about 16 hours. The reaction can be monitored by high performance liquid chromatography, and the time of reaction carefully coordinated with human insulin production.

Another facet of this invention, wholly unexpected, is the finding that the amount of Des-Thr(B30)-hI production can be further diminished by incorporation in the reaction mixture of one or more metal ions from another class of metals. This further improvement is particularly evident when the amount of the first metal ion is in the range of from about 0.1 mole to about 0.6 mole per mole of human insulin precursor. It is highly advantageous to add an amount of a metal ion of a metal selected from the group consisting of beryllium (Be), magnesium (Mg), calcium (Ca), strontium (Sr), barium (Ba), and radium (Ra). Preferably the ion will be that of calcium, barium, strontium, or magnesium, and, most preferably, will be that of calcium.

The amount of the second metal ion will range from about 0.5 mole to about 5 moles per mole of the human insulin precursor and, preferably, from about 1 mole to about 3 moles per mole of the human insulin precursor.

What has been most surprising about the use of a second metal ion as described in the foregoing is the fact that an ion of the second class, specifically calcium, is known to stabilize trypsin and when it is used in the absence of an ion of a metal of the first class, it has been noted that the production of Des-Thr(B30)-hI is actually increased.

Typically, the process of this invention is carried out by dissolving the human insulin precursor in an aqueous medium. The final mixture will generally be at a concentration of about 1 mM to about 3 mM and have a pH of about 8. Ion of a metal of the second class (if used) is then added. Typically, $CaCl_2$ will be added to a concentration of about 5 mM when the foregoing concentration of the human insulin precursor is used. An ion of a metal of the first class, typically Ni(II), is then added to a concentration of about 0.5 moles

per mole of the human insulin precursor. The pH of the mixture is adjusted to 7.3-7.5, and carboxypeptidase B (about 1:2,500 w/w human insulin precursor) is added followed by trypsin (about 1:12,500 w/w human insulin precursor). The reaction is allowed to proceed, the mixture being maintained at about 12 °C. Progress of the reaction is carefully monitored by high performance liquid chromatography.

The following examples are provided to demonstrate the efficacy of the process of this invention. They are not intended to be limiting upon the broad scope thereof.

Example 1 - Effect of Varying Trypsin and Carboxypeptidase B Concentrations.

Human proinsulin (hPI) was dissolved in 20 mM ethylene diamine (EDA) buffer, pH 7.0, at a concentration of 10.85 g/liter. The mixture was divided into two portions. To the first portion, porcine pancreatic carboxypeptidase B (CpB) was added to a final concentration of 3.74 mg/liter. This solution was divided into six one-milliliter aliquots, and bovine pancreatic trypsin, previously treated with tosyl-phenylalanyl chloromethyl ketone (trypsin-TPCK) was added at 1.0, 1.4, 1.8, 2.8, 3.6, and 5.4 mg/liter, respectively. Each of the samples was incubated for 8 hours at 23 °C. The Des-Thr(B30)-hI levels were determined by High Pressure Liquid Chromatography (HPLC) and are shown in Table 1.

The second portion of the hPI solution was divided into five one-milliliter aliquots. CpB was added to a concentration of 1.1, 1.5, 2.2, 3.7, and 5.4 mg/liter, respectively. Trypsin-TPCK then was added to each aliquot to a concentration of 2.71 mg/liter. Each of the samples was incubated for 8 hours at 23 °C. The results are presented in Table 2.

In both Tables 1 and 2, the amount of Des-Thr(B30)-hI is expressed as a percent of hI as determined by HPLC. As the data demonstrate, at fixed levels of CpB, Des-Thr(B30)-hI is reduced by decreasing levels of trypsin. Conversely, at fixed levels of trypsin, increasing levels of CpB lead to decreased levels of Des-Thr(B30)-hI.

Table 1

Effect of Increasing Levels of Trypsin on hPI Transformation

CpB, mg/liter	Trypsin, mg/liter	% Des-Thr(B30)-hI as % of hI
3.7	1.0	2.4
3.7	1.4	2.6
3.7	1.8	2.7
3.7	2.8	3.3
3.7	3.6	3.9
3.7	5.4	5.1

Table 2

Effect of Increasing Levels of CpB on hPI Transformation

CpB, mg/liter	Trypsin, mg/liter	% Des-Thr(B30)-hI as % of hI
1.1	2.71	4.8
1.5	2.71	4.0
2.2	2.71	4.1
3.7	2.71	3.4
5.4	2.71	2.6

Example 2 - Effect of Temperature on Des-Thr(B30)-hI Production.

hPI (60 mg) was dissolved in 20 mM ethylene diamine (6.0 ml), pH 7.5-8.0. Porcine carboxypeptidase B and bovine trypsin-TPCK were added sequentially to provide a substrate (hPI):enzyme ratio of 5000:1:1, w/w, for hPI:CpB:trypsin-TPCK. Two milliliter aliquots were incubated at 12, 24, and 37 °C for the lengths of time necessary to achieve maximum hI yield as measured by HpLC, i.e., 14, 6, and 4 hours, respectively. As shown in the results in Table 3, lower temperatures favored lower Des-Thr(B30)-hI formation.

Table 3

Temperature Effect

Incubation Temperature, °C	% Des-Thr(B30)-hI as % hI
12	4.4
24	>7
37	>9

Example 3 - Effect of Metals on Derivative Formation.

hPI (360 mg) was dissolved in 20 ml of 20 mM glycine, pH 7.65. The solution was divided into two 10.0 ml aliquots, and calcium ion at 5 mM was added to one aliquot. Each aliquot was divided further into three portions. A portion from the calcium ion-containing and one from the calcium ion-free aliquots then were treated as follows: For one set, zinc ion was added to give a 0.33 molar ratio relative to hPI. To another set, nickel ion was added to give a 0.36 molar ratio relative to hPI. Enzymes were added to all mixtures to provide the following weight ratios: hPI:CpB:trypsin-TPCK::13,500:5:1. The pH of each of the mixtures was adjusted to 7.65-7.7 and incubated at 12°C for 16 hours. The results, shown in Table 4, illustrate the effect of nickel and zinc in reducing the level of Des-Thr(B30)-hI formation. They further illustrate the enhancement of this effect by calcium.

Table 4

Effect of Metals on hPI Transformation

Metal Ion	Des-Thr(B30)-hI, as % hI
None	4.0
Ca	7.6
Zn	1.6
Ni	1.7
Zn+Ca	0.7
Ni+Ca	<0.2[1]

[1]Assay was less than detectable limit which was 0.20% of hI.

Example 4 - Effect of Varying Ni(II) Concentration on Derivative Formation in the hPI Conversion Reaction.

hPI (245 mg) was dissolved in 12.0 ml of 50 mM glycine, pH 7.4. Calcium ion was added from a 1 M $CaCl_2$ stock solution to yield a final Ca(II) concentration of 5 mM. Nickel(II) from a 0.11 M $NiCl_2$ stock

solution was added to 2 ml aliquots to give one sample each of a molar ratio to hPI of 0, 0.24, 0.37, 0.44, 0.51 and 0.58. CpB was added to each tube to yield 7.4 µg/ml (4.87 mg/ml stock) followed by addition of trypsin-TPCK to yield a final concentration of 2.96 µg/ml (1.0 mg/ml stock solution). The pH of all samples was adjusted to 7.40, and each was incubated at 12°C. Reactions were stopped after 12 hours, and levels of Des-Thr(B30)-hI and hI were measured. The results shown in Table 5, indicate that increased levels of nickel resulted in reduced production of Des-Thr(B30)-hI.

Table 5

Effect of Varying NI(II) Concentration

Molar Ratio, Ni(II)/hPI	% Des-Thr(B30)-hI, as % hI
0	7.6
0.24	1.9
0.37	0.61
0.44	0.72
0.51	0.33
0.58	0.28

Example 5 - Effect of Various Metal Cations on Derivative Formation in the hPI Conversion Reaction.

hPI (936 mg) was dissolved in 36 ml of 5 mM glycine, and the pH was adjusted to 7.8-8.0. Calcium ion was added as $CaCl_2$ (1 M stock) to 5 mM. Aliquots of 3 ml each were removed, and various metal ions were added at the concentration shown in Table 6. After equilibration at 12°C, enzymes were added to provide weight ratios as follows: hPI:CpB:trypsin-TPCK::13,500:5:1.

The samples were incubated at 12°C for 13 hours, and measured for hI and Des-Thr(B30)-hI. The results shown in Table 6 indicate that any of a wide range of metal ions are effective in reducing the production of Des-Thr(B30)-hI.

Table 6

Effect of Various Divalent Cations

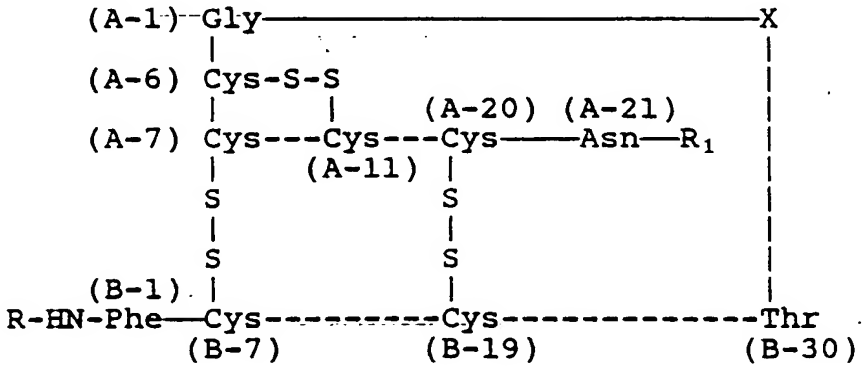
Divalent Metal Ion	Molar Ratio, M(II)/hPI	% Des-Thr(B30)-hI as % of hI
Zn	0.3	1.02
Zn	0.5	0.78
Ni	0.22	2.29
Ni	0.37	0.72
Co	0.26	2.35
Co	0.43	0.89
Cd	0.19	1.63
Cd	0.31	0.88
Cu	0.14	3.23
Cu	0.23	1.34

Example 6 - Large Scale Transformation of hPI Using Ni(II) and Ca(II).

hPI (448.5 g), dissolved in 15 mM glycine buffer, pH 7.4 (33.0 L), was cooled and maintained at 12 °C. Calcium(II) was added to 5 mM by addition of 1.0 M $CaCl_2$ stock solution (0.165 L). After stirring 10 minutes, nickel(II) was added to give a molar ratio Ni(II):hPI of 0.44:1 by addition of solid $NiCl_2 \cdot 6H_2O$ (5.0 g). The solution was stirred gently another 10 minutes, and CpB (36.8 ml, 179.4 mg) was added from a 4.87 mg/ml stock solution. Trypsin-TPCK (35.9 ml, 35.9 mg) then was added from a 1.0 mg/ml stock solution. The reaction reached completion in 10 hours as measured by maximal production of hI. At harvest, the mixture contained about 0.29% Des-Thr(B30)-hI, which approaches the detection limit of the method of detection of this compound.

Claims

1. A process for converting a human insulin precursor to human insulin, such precursor having the formula



in which R is hydrogen, a chemically or enzymatically cleavable amino acid residue, or a chemically or enzymatically cleavable peptide moiety having at least two amino acid residues;

R_1 is OH, Arg-Y, or Lys-Y in which Y is OH, an amino acid residue, or a peptide moiety having at least two amino acid residues;
the moiety from A-1 to A-21 is the human insulin A-chain; the moiety from B-1 to B-30 is the human insulin B-chain; and X is a moiety which is joined to the insulin A-chain at the amino group of A-1 and to the insulin B-chain at the carboxyl group of B-30, which moiety can be enzymatically cleaved from and without disruption of both the A-chain and the B-chain, which comprises treating such human insulin precursor with trypsin and carboxypeptidase B in an aqueous medium containing per mole of human insulin precursor from about 0.1 to about 10 moles of a first metal ion of those metals having Atomic Numbers 21 to 34, 39 to 52, 57 to 84, and 89 to 92, and a second metal ion of a metal selected from the group consisting of beryllium, magnesium, calcium, strontium, barium and radium.

2. Process of claim 1, in which the first metal ion is that of a metal selected from the group consisting of chromium, molybdenum, tungsten, mercury, antimony, bismuth, nickel, iron, cobalt, zinc, cadmium, copper, tin, lead, europium, uranium, platinum, and manganese.

3. Process of claim 2, in which the first metal ion is that of a metal selected from the group consisting of nickel, iron, cobalt, zinc, cadmium, copper, tin, lead, europium, uranium, platinum, and maganese.

4. Process of claim 3, in which the first metal ion is that of a metal selected from the group consisting of nickel, zinc, cobalt, and cadmium.

5. Process of any one of the claims 1 to 4, in which the human insulin precursor is present in the aqueous medium at a concentration of up to about 20 mM.

6. Process of claim 5, in which the human insulin precursor is present in the aqueous medium at a concentration of from about 1 mM to about 3 mM.

7. Process of any one of the claims 1 to 6, in which the first metal ion is present in an amount of from about 0.1 to about 2 moles per mole of human insulin precursor.

8. Process of claim 7, in which the first metal ion is present in an amount of from about 0.33 to about 0.6 moles per mole of human insulin precursor.

9. Process of any one of the claims 1 to 8, in which carboxypeptidase B is present in an amount on a weight basis, relative to the human insulin precursor, from about 1:10 to about 1:5,000.

10. Process of any one of the claims 1 to 9, in which trypsin is present in an amount on a weight basis, relative to the human insulin precursor, from about 1:20 to about 1:250,000.

11. Process of any one of the claims 1 to 10, in which the weight ratio of carboxypeptidase B to trypsin is from about 1:1 to about 10:1.

12. Process of any one of the claims 1 to 11, in which the first metal ion is that of a metal selected from the group consisting of nickel and zinc.

13. Process of claim 12, in which the first metal ion is nickel ion.

14. Process of any of claims 1 to 13, in which the second metal ion is that of a metal selected from the group consisting of calcium, barium, strontium, and magnesium.

15. Process of claim 14, in which the second metal ion is calcium ion.

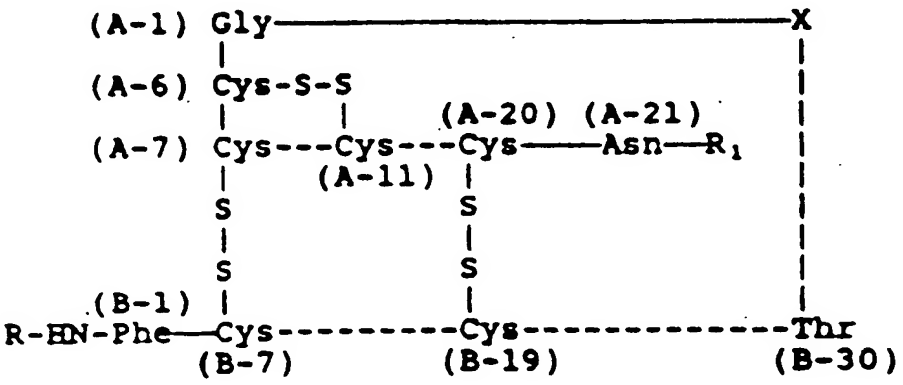
16. Process of either of claims 14 and 15, in which the second metal ion is present in an amount of from about 0.5 mole to about 5 moles per mole of the human insulin precursor.

17. Process of claim 16, in which the second metal ion is present in an amount of from about 1 mole to about 3 moles per mole of the human insulin precursor.

18. Process of any one of the claims 1 to 17, in which the human insulin precursor is human proinsulin.

Patentansprüche

1. Verfahren zur Umwandlung eines Humaninsulinvorläufers in Humaninsulin, wobei dieser Vorläufer die folgende Formel hat



worin R für Wasserstoff, einen chemisch oder enzymatisch spaltbaren Aminosäurerest oder einen chemisch oder enzymatisch spaltbaren Peptidrest mit zumindest zwei Aminosäureresten steht,

R_1 für OH, Arg-Y oder Lys-Y steht, worin Y für OH, einen Aminosäurerest oder einen Peptidrest mit mindestens zwei Aminosäureresten steht,

der Rest von A-1 bis A-21 die A-Kette von Humaninsulin ist, der Rest von B-1 bis B-30 die B-Kette von Humaninsulin ist, und X ein Rest ist, der an die A-Kette von Insulin an die Aminogruppe von A-1 und an die B-Kette von Insulin an die Carboxylgruppe von B-30 gebunden ist, wobei dieser Rest ohne Zerstörung sowohl der A-Kette als auch der B-Kette enzymatisch abgespalten werden kann, gekennzeichnet durch eine Behandlung eines solchen Humaninsulinvorläufers mit Trypsin und Carboxypeptidase B in einem wäßrigen Medium, das pro Mol Humaninsulinvorläufer etwa 0,1 bis etwa 10 Mol eines ersten Metallions von Metallen mit den Atomzahlen 21 bis 34, 39 bis 52, 57 bis 84 und 89 bis 92, und ein zweites Metallion eines Metalls enthält, ausgewählt aus der aus Beryllium, Magnesium, Calcium, Strontium, Barium und Radium bestehenden Gruppe.

2. Verfahren nach Anspruch 1, worin das erste Metallion von einem Metall stammt, ausgewählt aus der aus Chrom, Molybdän, Wolfram, Quecksilber, Antimon, Wismuth, Nickel, Eisen, Cobalt, Zink, Cadmium, Kupfer, Zinn, Blei, Europium, Uran, Platin und Mangan bestehenden Gruppe.

3. Verfahren nach Anspruch 2, worin das erste Metallion von einem Metall stammt, ausgewählt aus der aus Nickel, Eisen, Cobalt, Zink, Cadmium, Kupfer, Zinn, Blei, Europium, Uran, Platin und Mangan bestehenden Gruppe.

4. Verfahren nach Anspruch 3, worin das erste Metallion von einem Metall stammt, ausgewählt aus der aus Nickel, Zink, Cobalt und Cadmium bestehenden Gruppe.

5. Verfahren nach einem der Ansprüche 1 bis 4, worin der Humaninsulinvorläufer im wäßrigen Medium in einer Konzentration bis zu etwa 20 mM vorhanden ist.

6. Verfahren nach Anspruch 5, worin der Humaninsulinvorläufer im wäßrigen Medium in einer Konzentration von etwa 1 mM bis etwa 3 mM vorhanden ist.

7. Verfahren nach einem der Ansprüche 1 bis 6, worin das erste Metallion in einer Menge von etwa 0,1 bis etwa 2 Mol pro Mol Humaninsulinvorläufer vorhanden ist.

8. Verfahren nach Anspruch 7, worin das erste Metallion in einer Menge von etwa 0,33 bis etwa 0,6 Mol pro Mol Humaninsulinvorläufer vorhanden ist.

9. Verfahren nach einem der Ansprüche 1 bis 8, worin Carboxypeptidase B auf Gewichtsbasis in einer Menge relativ zum Humaninsulinvorläufer von etwa 1:10 bis etwa 1:5 000 vorhanden ist.

10. Verfahren nach einem der Ansprüche 1 bis 9, worin Trypsin auf Gewichtsbasis in einer Menge relativ zum Humaninsulinvorläufer von etwa 1:20 bis etwa 1:250 000 vorhanden ist.

11. Verfahren nach einem der Ansprüche 1 bis 10, worin das Gewichtsverhältnis von Carboxypeptidase B zu Trypsin etwa 1:1 bis etwa 10:1 beträgt.

12. Verfahren nach einem der Ansprüche 1 bis 11, worin das erste Metallion das eines Metalls ist, ausgewählt aus der aus Nickel und Zink bestehenden Gruppe.

13. Verfahren nach Anspruch 12, worin das erste Metallion das Nickelion ist.

14. Verfahren nach einem der Ansprüche 1 bis 13, worin das zweite Metallion von einem Metall stammt, ausgewählt aus der aus Calcium, Barium, Strontium und Magnesium bestehenden Gruppe.

15. Verfahren nach Anspruch 14, worin das zweite Metallion das Calciumion ist.

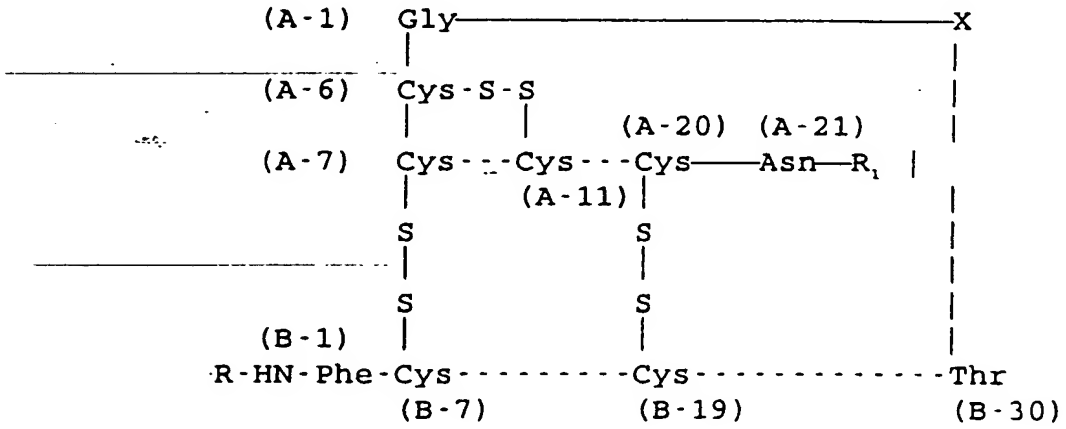
16. Verfahren nach einem der Ansprüche 14 oder 15, worin das zweite Metallion in einer Menge von etwa 0,5 Mol bis etwa 5 Mol, pro Mol Humaninsulinvorläufer vorhanden ist.

17. Verfahren nach Anspruch 16, worin das zweite Metallion in einer Menge von etwa 1 Mol bis etwa 3 Mol pro Mol Humaninsulinvorläufer vorhanden ist.

18. Verfahren nach einem der Ansprüche 1 bis 17, worin der Humaninsulinvorläufer humanes Proinsulin ist.

Revendications

1. Procédé pour transformer en insuline humaine un précurseur de l'insuline humaine, ledit précurseur répondant à la formule



dans laquelle R représente un atome d'hydrogène, un résidu d'acide aminé séparable par voie chimique ou par voie enzymatique, ou encore une fraction peptidique séparable par voie chimique ou par voie enzymatique, possédant au moins deux résidus d'acides aminés;

R1 représente OH, Arg-Y ou Lys-Y où Y représente OH, un résidu d'acide aminé ou une fraction peptidique possédant au moins deux résidus d'acides aminés; la fraction de A-1 à A-21 représente la chaîne A de l'insuline humaine; la fraction de B-1 à B-30 représente la chaîne B de l'insuline humaine; et X représente la fraction qui est jointe à la chaîne A de l'insuline au groupe amino de A-1 et à la chaîne B de l'insuline au groupe carboxyle de B-30, ladite fraction pouvant être séparée par voie enzymatique à la fois de la chaîne A et de la chaîne B sans rupture de ces dernières, qui comprend le

fait de traiter ledit précurseur de l'insuline humaine avec de la trypsine et de la carboxypeptidase B dans un milieu aqueux contenant, par mole du précurseur de l'insuline humaine, d'environ 0,1 à 10 moles d'un premier ion métallique des métaux possédant un nombre atomique de 21 à 34, de 39 à 52, de 57 à 84 et de 89 à 92, et d'un second ion métallique d'un métal choisi parmi le groupe constitué par le béryllium, le magnésium, le calcium, le strontium, le baryum et le radium.

2. Procédé selon la revendication 1, dans lequel le premier ion métallique est celui d'un métal choisi parmi le groupe constitué par le chrome, le molybdène, le tungstène, le mercure, l'antimoine, le bismuth, le nickel, le fer, le cobalt, le zinc, le cadmium, le cuivre, l'étain, le plomb, l'europium, l'uranium, le platine et le manganèse.

3. Procédé selon la revendication 2, dans lequel le premier ion métallique est celui d'un métal choisi parmi le groupe constitué par le nickel, le fer, le cobalt, le zinc, le cadmium, le cuivre, l'étain, le plomb, l'europium, l'uranium, le platine et le manganèse.

4. Procédé selon la revendication 3, dans lequel le premier ion métallique est celui d'un métal choisi parmi le groupe constitué par le nickel, le zinc, le cobalt et le cadmium.

5. Procédé selon l'une quelconque des revendications 1 à 4, dans lequel le précurseur de l'insuline humaine est présent dans le milieu aqueux à une concentration allant jusqu'à environ 20 mM.

6. Procédé selon la revendication 5, dans lequel le précurseur de l'insuline humaine est présent dans le milieu aqueux à une concentration d'environ 1 mM à environ 3 mM.

7. Procédé selon l'une quelconque des revendications 1 à 6, dans lequel le premier ion métallique est présent en une quantité d'environ 0,1 à environ 2 moles par mole du précurseur de l'insuline humaine.

8. Procédé selon la revendication 7, dans lequel le premier ion métallique est présent en une quantité d'environ 0,33 à environ 0,6 mole par mole du précurseur de l'insuline humaine.

9. Procédé selon l'une quelconque des revendications 1 à 8, dans lequel la carboxypeptidase B est présente en une quantité, sur une base pondérale, par rapport au précurseur de l'insuline humaine, d'environ 1:10 à environ 1:5.000.

10. Procédé selon l'une quelconque des revendications 1 à 9, dans lequel la trypsine est présente en une quantité, sur une base pondérale, par rapport au précurseur de l'insuline humaine, d'environ 1:20 à environ 1:250.000.

11. Procédé selon l'une quelconque des revendications 1 à 10, dans lequel le rapport pondéral de la carboxypeptidase B à la trypsine est d'environ 1:1 à environ 10:1.

12. Procédé selon l'une quelconque des revendications 1 à 11, dans lequel le premier ion métallique est celui d'un métal choisi parmi le groupe constitué par le nickel-et-le-zinc.

13. Procédé selon la revendication 12, dans lequel le premier ion métallique est un ion de nickel.

14. Procédé selon l'une quelconque des revendications 1 à 13, dans lequel le second ion métallique est celui d'un métal choisi parmi le groupe constitué par le calcium, le baryum, le strontium et le magnésium.

15. Procédé selon la revendication 14, dans lequel le second ion métallique est un ion calcium.

16. Procédé selon l'une quelconque des revendications 14 et 15, dans lequel le second ion métallique est présent en une quantité d'environ 0,5 mole à environ 5 moles par mole du précurseur de l'insuline humaine.

17. Procédé selon la revendication 16, dans lequel le second ion métallique est présent en une quantité d'environ 1 mole à environ 3 moles par mole du précurseur de l'insuline humaine.

18. Procédé selon l'une quelconque des revendications 1 à 17, dans lequel le précurseur de l'insuline humaine est la proinsuline humaine.